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5-Arylidene-2,4-thiazolidinediones as inhibitors of protein tyrosine phosphatases

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Abstract—4-(5-Arylidene-2,4-dioxothiazolidin-3-yl)methylbenzoic acids (**2**) were synthesized and evaluated in vitro as inhibitors of PTP1B and LMW-PTP, two protein tyrosine phosphatases (PTPs) which act as negative regulators of the metabolic and mitotic signalling of insulin. The synthesis of compounds **2** represents an example of utilizing phosphotyrosine-mimetics to identify effective low molecular weight nonphosphorus inhibitors of PTPs. Several thiazolidinediones **2** exhibited PTP1B inhibitory activity in the low micromolar range with moderate selectivity for human PTP1B and IF1 isoform of human LMW-PTP compared with other related PTPs.

1. Introduction

Reversible protein tyrosine phosphorylation regulates a variety of cellular functions, including proliferation, differentiation, migration, metabolism, gene transcription, cell–cell communication, ion channel activity, immune response and apoptosis/survival decisions. It is controlled by the coordinated action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).[1–6] Some members of the PTP family, which comprises more than 100 enzymes, exhibit substrate specificity. They have also been recognized to exert positive and negative critical effects on specific signalling pathways and thus play complex and crucial physiological roles in most mammalian cells.[1–4,6–8] As observed with PTKs, the deregulation of PTP activity contributes to the development of many human pathologies, including cancer, diabetes, inflammation and neurodegenerative diseases.[1–4,9] Moreover, tyrosine phosphorylation is critical to glucose uptake and metabolism in insulin-sensitive tissues.[4,9–11]

PTP1B is an intracellular PTP and is a key negative regulator of the insulin signalling pathway. It acts by dephosphorylation of specific phosphotyrosine (pTyr) residues on the insulin receptor and insulin receptor substrate proteins.[1–3,9–12] A high correlation between cellular levels of PTP1B and insulin resistance has been found. In fact, PTP1B knockout mice displayed both increased insulin sensitivity in liver and in skeletal muscles and resistance to obesity, without abnormalities in growth or fertility or increased incidence of cancer. This indicates that PTP1B functions are tissue specific.[9,13]

Other PTPs, such as leukocyte antigen related PTP (LAR-PTP), SHP-2, PTPα and low molecular weight PTP (LMW-PTP), appear to be involved in the regulation of insulin signalling. It is probable that they control this pathway in cell types which are unaffected by PTP1B.[9] However, for some their physiological role remains unclear.

One of these, LMW-PTP, possesses a phosphate binding loop structurally identical to that of PTP1B and has been recognized as a negative regulator of insulin-medi-

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ated mitotic and metabolic signalling.[14,15] It has also been reported that LMW-PTP can interact with other receptor tyrosine kinases, including EGFR,[16] PDGFR,[17] fibroblast growth factor receptor[18] and Ephrin receptor.[19]

Other reports demonstrated that the overexpression of PTP1B and LMW-PTP is sufficient to transform non-transformed cells. Indeed, high levels of these enzymes have been detected in some types of human tumours.[4,20–22] The overexpression of PTPα also produces cell transformation in rat embryonic fibroblasts.[23] Tartaglia identified some PTPN11 mutations that are associated with some forms of leukaemia,[24] while Saha found that PRL-3 PTP is clearly associated with colorectal cancer metastasis.[25] Thus, like PTKs, PTPs can also play an oncogenic role.

In light of the above, the inhibition of PTP1B, as well as of LMW-PTP, can be considered an attractive approach in the design of new therapeutic agents for the treatment of type 2 diabetes (DM2) and obesity. These two related pathologies are often associated with complex metabolic disorders known as metabolic syndrome (MS). Given the epidemic proportions of these diseases, it is crucial to discover new drugs that are able to counteract both insulin resistance and weight gain, as these are closely connected with the onset and progression of both DM2 and MS.[1,4]

At the same time, PTP1B and LMW-PTP are of interest as novel targets in the search for new antitumoral drugs.

Indeed, there is a great deal of research in progress exploring PTP1B and LMW-PTP inhibitors as potential therapeutic agents.[26,27] Over the last decade, numerous different PTP1B inhibitors have been reported. However, none have passed clinical trials to date. This failure in trials is generally due either to side effects or a low rate of in vivo efficacy. These could be the consequence of either scarce selectivity or limited cell permeability.[4]

The specific inhibition of a single enzyme of the PTP family is often difficult to achieve because the active site is highly conserved among such enzymes. However, in the case of PTP1B, the identification of a second noncatalytic arylphosphate binding site, which is not present in all PTPs, has provided a further structural basis for targeted drug design.[11,28,29] On the other hand, although cell permeable inhibitors of PTP1B have been reported, [26d,26g,30,31] efforts to obtain selective inhibitors of this phosphatase have often resulted in the synthesis of peptidic or large highly charged molecules with high in vitro potency but potentially low bioavailability. Thus the discovery of pharmaceutically appropriate PTP1B inhibitors is still a great challenge.

The current thrust of research in this field is the identification of low molecular weight, nonphosphorus and nonpeptide inhibitors that are potentially endowed with favourable pharmacokinetics. A useful and commonly adopted strategy in the design of PTP1B inhibitors is to insert nonhydrolysable pTyr-mimetic groups that are able to replicate the interactions of pTyr residue with the active site of the enzyme onto different optimal templates.[26g,26i,26j,32–37] Replacing the phosphate group of pTyr with bioisosteric monoanionic groups, such as carboxylates, is considered a valid method to obtain inhibitors with low polarity.[26c,30,31,34] Indeed, several carboxylic acids with in vitro PTP1B inhibitory properties and good cellular activity or oral bioavailability have been reported.[26b,26d,30,31,36] Allosteric PTP1B inhibitors are also known.[38]

We have been searching for new 2,4-thiazolidinediones (2,4-TZDs) as potential agents for the treatment of DM2 and its complications over the last few years.[39] 2,4-TZDs have excited considerable interest as antihyperglycaemic compounds[40] and aldose reductase inhibitors.[41] Some of them (pioglitazone, rosiglitazone) are insulin-sensitizing agents acting as peroxisome proliferator-activated receptor γ (PPAR γ) agonists,[40d] and have recently been marketed for the treatment of DM2. In addition some 2,4-TZDs have proved to be PTP1B inhibitors.[42]

In this paper, we report on the synthesis and biological activity of a series of 4-(5-arylidene-2,4-dioxothiazolidin-3-yl)methylbenzoic acids (**2a**–**j**) which have been designed as PTP1B and LMW-PTP inhibitors.

The starting point for our PTP1B inhibitor design was to insert the *p*-methylbenzoic acid residue onto N-3 of the thiazolidinedione scaffold. It has already been suggested that benzoic acid could act as a nonphosphorus-containing pTyr-mimic.[43] It is likely that its carboxylic group mimics the interactions of the phosphate group of pTyr with Arg221 in the PTP1B catalytic site while the benzene ring might interact with Phe182 and Tyr46.

The lipophilic 5-arylidene moiety of acids **2** containing one or two aromatic rings was also inserted in an attempt to improve the stability of the enzyme/inhibitor complex. This may occur due to hydrophobic contacts with the lipophilic residues which surround the active pTyr-binding site.

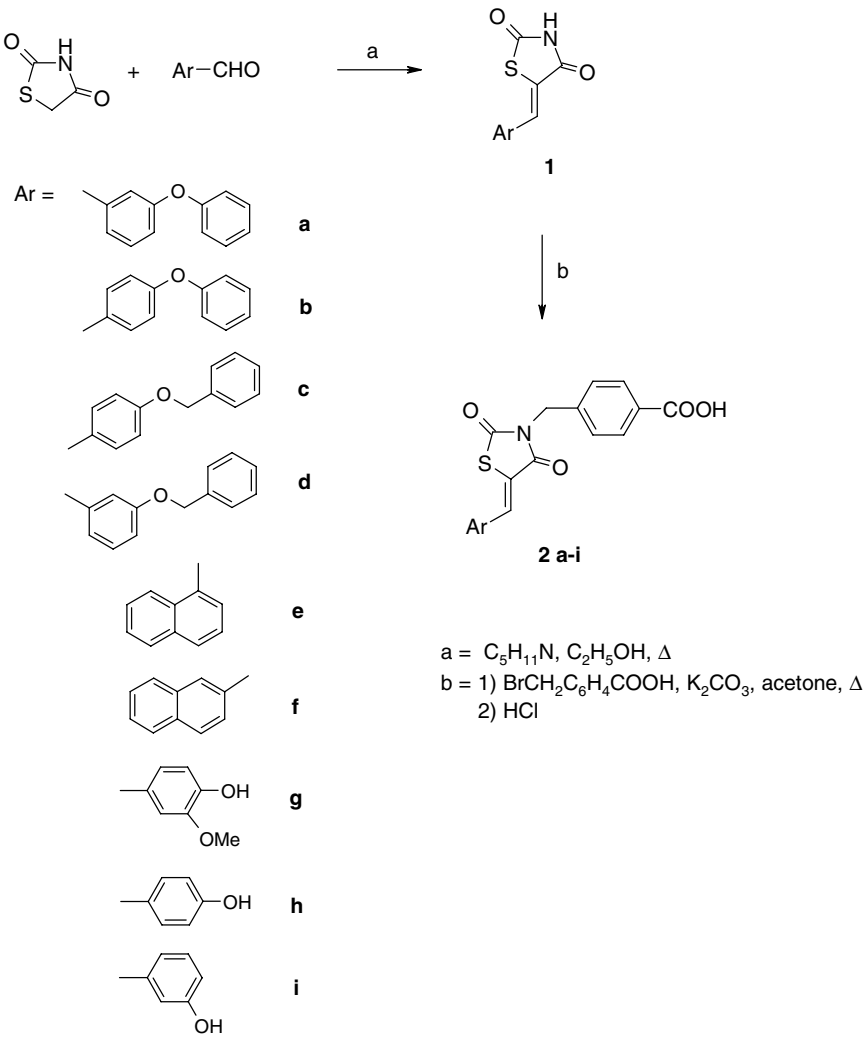
Compounds **2a**–**j** were tested for their in vitro inhibitory activity not only against human PTP1B but also the two active isoforms of human LMW-PTP (IF1 and IF2) as well as the *S. cerevisiae* LMW-PTP (Ltp1). Compounds **2b** and **2c** were also selected to be assayed against other related PTPs (TC-PTP, LAR-PTP, PTPβ, YopH).

Docking simulations into the PTP1B active site allowed the results obtained to be rationalised.

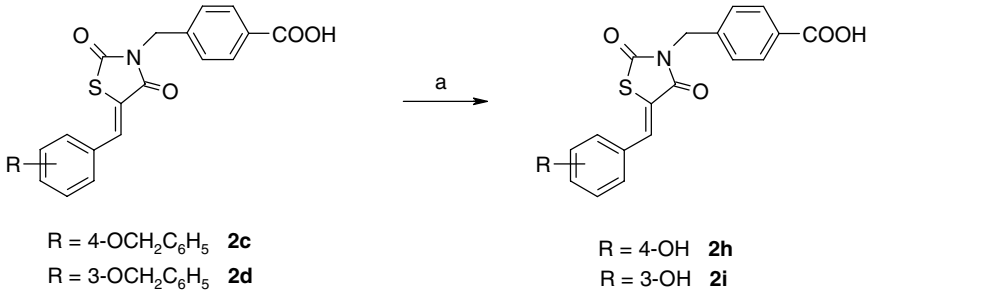
2. Chemistry

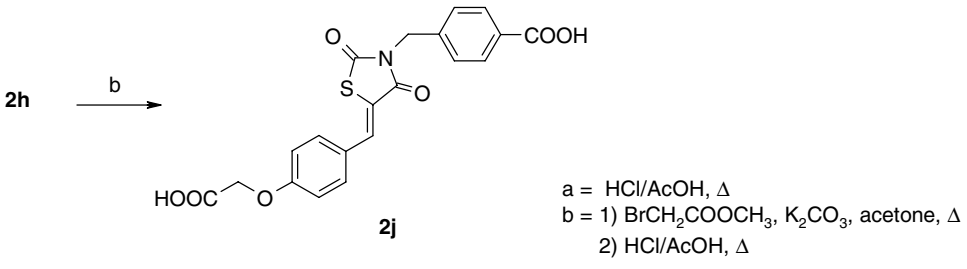
The general method for the synthesis of (*Z*)-4-(5-arylidene-2,4-dioxothiazolidin-3-yl) methylbenzoic acids (**2**) is depicted in Scheme 1.

(*Z*)-5-Arylidene-2,4-thiazolidinediones **1** were obtained by the condensation of commercially available



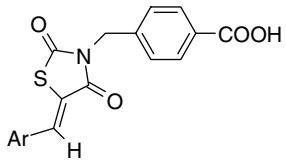
Scheme 1.

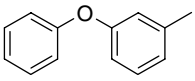
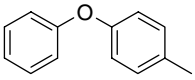
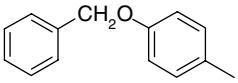
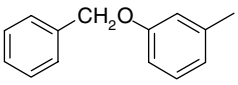
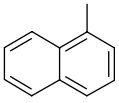
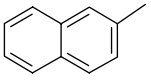
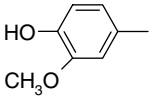
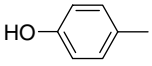
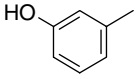
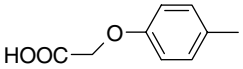




Scheme 2.

Table 1. In vitro inhibitory activity of compounds **2a–j** against human PTP1B, human LMW-PTP (IF1 and IF2 isoforms) and *S. cerevisiae* LMW-PTP (Ltp1)[a]



Ar	Compound	IC_{50} (μM)			
		PTP1B	IF1	IF2	Ltp1
	**2a**	18.0 ± 1.0	37.0 ± 11.0	130.0 ± 17.0	483.0 ± 132.0
	**2b**	2.8 ± 0.2	0.9 ± 0.3	13.0 ± 4.0	34.0 ± 4.0
	**2c**	1.6 ± 0.2	2.5 ± 0.2	5.6 ± 0.4	15.0 ± 1.0
	**2d**	1.1 ± 0.1	7.1 ± 1.4	18.4 ± 1.0	19.7 ± 4.5
	**2e**	5.5 ± 1.0	122.0 ± 37.0	138.0 ± 37.0	99.0 ± 7.0
	**2f**	6.5 ± 0.3	12.0 ± 1.0	92.0 ± 5.0	149.0 ± 8.0
	**2g**	2.6 ± 0.4	9.1 ± 1.6	26.6 ± 1.5	389 ± 107
	**2h**	10.0 ± 1.0	30.0 ± 2.0	85.0 ± 8.0	188.0 ± 15.0
	**2i**	10.2 ± 1.1	85.7 ± 21	112.4 ± 9	1395 ± 220
	**2j**	85.0 ± 24.0	219.0 ± 50.0	1642.0 ± 75.0	>450

[a] IC_{50} values were determined by regression analyses and expressed as means ± SE of three replicates.

2,4-thiazolidinedione with suitable aromatic aldehydes in refluxing ethanol in the presence of piperidine, as already reported[39a,b] (Scheme 1).

The reaction between 2,4-thiazolidinediones **1** and 4-(bromomethyl)benzoic acid, using potassium carbonate as base in refluxing acetone, followed by a workup in acidic medium and recrystallization from methanol, provided pure acids **2** (Scheme 1).

The synthesis of 4-[5-(4-hydroxy-3-methoxybenzylidene)-2,4-dioxothiazolidin-3-yl] methylbenzoic acid (**2g**) was carried out by adding 4-(bromomethyl)benzoic acid in small amounts to a solution of **1g** and potassium carbonate in acetone over a 2-h period in order to prevent the nucleophilic attack of 4-(bromomethyl)benzoic acid by the hydroxy group. After refluxing the mixture for 48 h and crystallization from methanol the desired acid **2g** was obtained as a single product.

4-[5-(4-Hydroxybenzylidene)-2,4-dioxothiazolidin-3-yl] methylbenzoic acid (**2h**) and its 5-(3-hydroxybenzylidene) substituted isomer **2i** were prepared by the acidic hydrolysis of 5-(4-benzyloxybenzylidene)- and 5-(3-benzyloxybenzylidene) substituted analogues **2c** and **2d**, respectively (Scheme 2). As for **2g**, acids **2h** and **2i** were also obtained by the reaction of **1h** and **1i** with 4-(bromomethyl)benzoic acid (Scheme 1), but with slightly lower yields.

The O-alkylation of **2h** with methyl bromoacetate in the presence of potassium carbonate in refluxing acetone followed by acidic hydrolysis provided 4-[5-(4-carboxymethoxybenzylidene)-2,4-dioxothiazolidin-3-yl]methylbenzoic acid (**2j**) (Scheme 2).

The structures of compounds **2** were assigned on the basis of their analytical and spectroscopic data (IR, ^{1}H and ^{13}C NMR). The ^{1}H NMR spectra showed a singlet in the range from 4.75 to 4.93 ppm attributable to N–CH_2 resonance, which was diagnostic. In ^{13}C NMR spectra, the carbon atom of the same methylene group resonated at 44.0–44.9 ppm as a triplet. In addition, in ^{13}C NMR spectra, besides two signals due to the resonances of 2- and 4-carbonylic groups of the thiazolidinedione ring at 164.8–168.9 ppm, another singlet attributable to the resonance of the carboxylic carbon was present in the same range.

Analogously to the NMR spectra of parent compounds **1**, which were obtained only as *Z* isomers,[39a] the ^{1}H NMR spectra of acids **2** showed only one singlet attributable to the resonance of the 5-methylidene proton in the range from 7.88 to 8.22 ppm. In ^{13}C NMR spectra a doublet at 131.0–134.2 ppm was due to the 5-methylidene carbon, while thiazolidinedione C-5 resonated as a singlet at 116.4–124.7 ppm.

The structure of acids **2** was also confirmed by means of their IR spectra. These showed a very broad band in the region of 3450–2450 cm^{-1} attributable to the stretching of carboxylic OH.

3. Results and discussion

Compounds **2a–j** were evaluated in vitro for their inhibitory activity against recombinant human PTP1B as well as the two active isoforms of human LMW-PTP (IF1 and IF2) and *S. cerevisiae* LMW-PTP (Ltp1). *p*-Nitrophenylphosphate (*p*-NPP) was used as a substrate.

It is known that interactions between pTyr-flanking residues and sub-pockets bordering the PTP1B catalytic site contribute to high affinity substrate binding.[1,44] Thus, it has been suggested that interactions with these adjacent subsites could also enhance inhibitor affinity and selectivity.[1,29] We therefore decided to vary the 5-arylidene moiety in order to evaluate its influence on the inhibitory properties of acids **2**, whilst keeping the 2,4-TZD framework and the pTyr-mimetic group on N-3 constant.

We first synthesized 4-(5-arylidene-2,4-dioxothiazolidin-3-yl)methylbenzoic acids with a 5-arylidene moiety containing two aromatic rings (compounds **2a–f**). It was thought this might stabilize the complex with the enzyme through hydrophobic interactions with the active site and surrounding subpockets, as this has often been observed in PTP1B/inhibitor complexes.[26c,31,34,36,43] Subsequently, we also prepared analogues which had a 5-benzylidene moiety with hydrophilic H-donor substituents (compounds **2g–j**). This was done because the enzyme/inhibitor complex stability might be increased by means of further interactions via hydrogen bonds due to the presence of hydrophilic residues in both catalytic and vicinal binding sites of PTP1B.[26c,31,36,44,45]

The results of the in vitro inhibition assays, reported in Table 1, did in fact indicate that the inhibitory effects and selectivity of acids **2** differed markedly depending on the 5-arylidene moiety.

Acids **2b** and **2c**, bearing a phenoxy and a benzyloxy group in the *para* position of the 5-benzylidene ring, respectively, performed well as inhibitors of both human PTP1B and human LMW-PTP isoforms and exhibited IC_{50} values in the low micromolar range. In contrast, they were poor Ltp1 inhibitors (Table 1). Compond **2b** in particular was found to be fairly selective, since it was from 5- to 38-fold more effective against IF1 (IC_{50} = 0.9 μM) and PTP1B (IC_{50} = 2.8 μM) than against IF2 (IC_{50} = 13 μM) and Ltp1 (IC_{50} = 34 μM).

The displacement of the 4-benzyloxy group to position 3 of the 5-benzylidene moiety yielded compound **2d** and this exhibited an appreciable PTP1B inhibitory effect, similar to that of **2c**, but with lower activity levels against IF1, IF2 and Ltp1 (Table 1). In contrast, the displacement of the 4-phenoxy group to *meta* position resulted in a reduction of activity. In fact, acid **2a** was shown to be about 6-fold less active than its isomer **2b** towards PTP1B. The decrease in inhibitory effectiveness was even more significant (from 10 to 40 times) against human LMW-PTP isoenzymes (Table 1).

The condensation of a further benzene ring to the 5-benzylidene group led to 5-naphthalen-1-ylmethylene and 5-naphthalen-2-ylmethylene substituted derivatives (compounds **2e** and **2f**). These were only fairly selective micromolar inhibitors of PTP1B, and their inhibitory effects against human LMW-PTP isoforms as well as Ltp1 were moderate or poor (Table 1). In particular, **2e** and **2f** proved to be 18- to 25-fold and 2- to 23-fold more selective for PTP1B, respectively (Table 1).

The removal of the additional aromatic ring and its replacement with a hydrophilic group (compounds **2g–j**) resulted in either a slight or a marked reduction of the inhibitory effect depending on the substituents on the 5-benzylidene ring. The removal of the benzyl group converted the most potent PTP1B inhibitors among the tested acids **2**, that is, 5-(4-benzyloxybenzylidene)- and 5-(3-benzyloxybenzylidene) substituted acids **2c** and **2d**, into the hydroxybenzylidene substituted analogues **2h** and **2i**, respectively. These were about 10-fold less active against PTP1B and from 6 to 70 times less effective towards the other PTPs tested. In fact, **2h** and **2i** proved to be 3- to 19-fold and 8- to 137-fold more selective for PTP1B, respectively (Table 1).

The insertion of a methoxy group in the *meta* position of the 5-(4-hydroxybenzylidene) moiety of **2h** led to derivative **2g** and provided a 4-fold gain in the PTP1B inhibitory effect. Compound **2g** was also about 3 times more active against both human LMW-PTP isoforms and two fold less effective against Ltp1 than **2h** (Table 1).

In contrast, the replacement of the 4-hydroxy group with a carboxymethoxy one (compound **2j**) resulted in

a significant reduction of activity against all screened PTPs. In fact, acid **2j** was the least effective PTP inhibitor among the tested compounds **2**.

A kinetic study was performed in order to shed light on the inhibitory mechanism of compounds **2**. It revealed that acids **2** behaved as competitive and reversible inhibitors of all the PTPs tested. This finding was to be expected given that they all are nonhydrolysable pTyr-mimetic compounds. Figure 1 shows the double reciprocal plots of the three most active PTP1B inhibitors (**2b**, **2c**, **2d**) out of acids **2**.

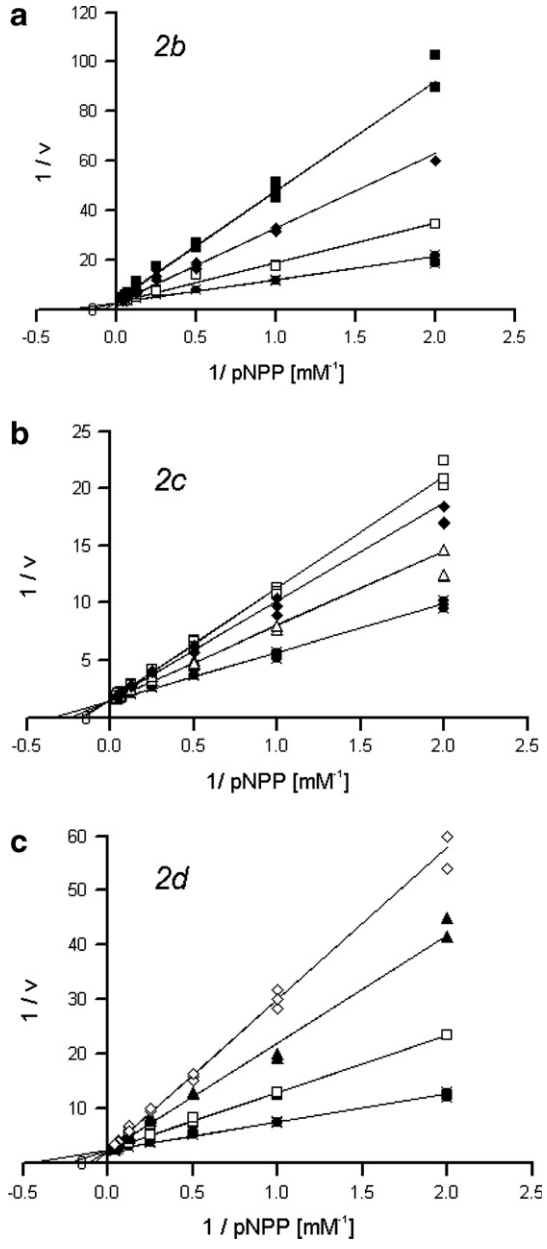


Figure 1. Lineweaver–Burk plots for the three best PTP1B inhibitors. The inhibitor concentration was as follows: top panel, (●) no inhibitor; (□) 1 μM; (♦) 2 μM; (■) 3 μM. Middle panel, (●) no inhibitor; (△) 0.5 μM; (♦) 0.75 μM; (□) 1 μM. Bottom panel, (●) no inhibitor; (□) 1 μM; (▲) 2 μM; (◊) 3 μM. All tests were performed with the enzyme PTP1B. The symbol v in the ordinate axis indicates the initial substrate hydrolysis rate expressed as arbitrary units.

The capability of compounds **2** to inhibit peptide dephosphorylation by PTP1B was assessed by using a peptide corresponding to the sequence 1142–1153 of the insulin receptor. The IC_{50} values of the most active PTP1B inhibitors **2b** (23.0 ± 1.3 μM), **2c** (7.6 ± 1.7 μM), and **2d** (28.8 ± 2.4 μM) were calculated measuring the dephosphorylation rates at increasing inhibitor concentrations, in the presence of 170 μM phosphopeptide substrate (a concentration corresponding to the K_m value for PTP1B).

The most effective inhibitors of both human PTP1B and LMW-PTP out of the tested acids **2**, compounds **2b** and **2c**, were further assayed against certain other related PTPs (Table 2).

They proved to be equally effective as inhibitors of both PTP1B and T-cell protein tyrosine phosphatase (TC-PTP) (Table 2). These two enzymes are highly homologous (74% sequence identity) and selectivity for only one of them is generally difficult to achieve.[26k,29,37,45]

In contrast, the inhibitory effects of **2b** and **2c** markedly decreased (from 10 to 20 times) against LAR, a transmembrane receptor-like PTP. LAR has also been recognized as a negative regulator of insulin signalling. However, its deficiency leads to neuronal defects and impaired development of mammary glands in mice.[9] Thus, an inhibitor that is selective to PTP1B rather than LAR would be desirable. Moreover, since LAR does not contain the second arylphosphate binding site of PTP1B, these inhibition data seem to suggest that the residues lining this secondary pocket should play a role in the binding mode of inhibitors **2**. This hypothesis was supported by the significant inhibitory activity of **2b** and **2c** against YopH (Table 2). The latter PTP is a virulence factor that is essential for the pathogenicity of *Yersinia* genus and it possesses a second phosphate binding site, just as found in PTP1B.[46]

We also observed different inhibitory effects of compounds **2b** and **2c** towards the human LMW-PTP isoenzymes which differ only in the sequence 40–73.[47] In particular, the inhibitory activity of **2b** towards IF1 was 14-fold higher than for IF2, while inhibitory activity of **2c** towards IF1 was only 2-fold higher than for IF2. This behaviour could be due to the structural differences

Table 2. Comparison of the inhibitory activity of **2b** and **2c** against protein phosphatases[a]

PTPs	2b	2c
PTP1B	2.8 ± 0.2	1.6 ± 0.2
IF1	0.9 ± 0.3	2.5 ± 0.2
IF2	13.0 ± 4.0	5.6 ± 0.4
Ltp1	34.0 ± 4.0	15.0 ± 1.0
TC-PTP	2.8 ± 0.3	1.3 ± 0.2
LAR-PTP	24.6 ± 4.1	27.0 ± 6.6
PTPβ	1.1 ± 0.1	0.4 ± 0.1
YopH	1.0 ± 0.1	0.3 ± 0.1

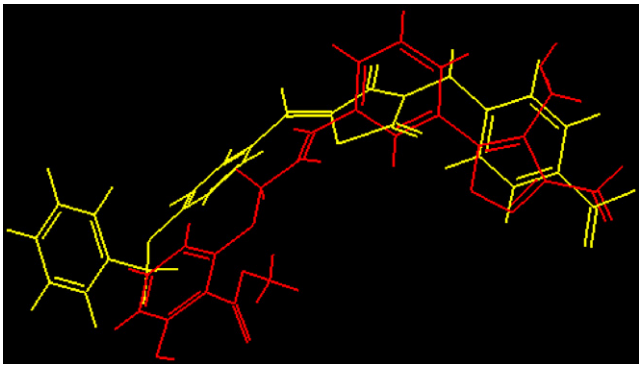
[a] IC_{50} values were determined by regression analyses and expressed as means ± SE of three replicates.

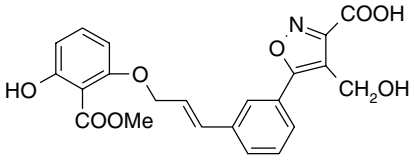
in the alternative sequences which are known to participate in the formation of a loop situated very close to the active site.[11,29] In addition, greater differences are observed when comparing the human isoenzymes with the yeast Ltp1 that shares about 40% sequence identity with the mammalian enzymes.[48]

4. Molecular docking

Compounds **2b** and **2c** were selected for docking experiments into the PTP1B active site. Compound **2j** was also included in this investigation in order to find a possible explanation for its lower inhibitory activity.

The LigandFit module implemented in the Cerius[2] software package[49] was used for the docking procedure.





CAS 745079-21-4

Figure 2. Compound **2c** (yellow) overlaid with bound ligand (red) from 1XBO.

PDB entry 1XBO[31] was chosen as a starting point for the docking process. Analysis of the cocrystallized ligand (CAS 745079-21-4) guided this choice. 745079-21-4 is similar to acids **2b** and **2c** in size and topology (Fig. 2) and is also active at low micromolar doses. The resolution of this complex is 2.5 Å. In LigandFit the protein was prepared for docking (removal of ligand and water molecules, addition of hydrogens) using the cff1.02 force field. Compounds **2b**, **2c** and **2j** were imported as minimised structures from the software Catalyst[50] as sd files.

On completion of the flexible docking process, the resulting conformations/poses of the ligands in the binding site of 1XBO were studied (Figs. 3–5). A dense network of hydrogen bonds between the carboxylic group of inhibitors and Arg221, Gly220, Ser216, Cys215, Gly218, Ala 217 was observed as well as hydrophobic interactions between the phenyl rings of the ligands and surrounding lipophilic amino acid residues (e.g., with Phe182). A strong driving force for binding was the charge-charge interaction between the carboxylate group of the ligand and Arg221. This interaction pattern is common in many known PTP1B/inhibitor complexes.[26d,28,29,31,33,34,43]

Furthermore, the ligands reached into the secondary noncatalytic binding pocket and therefore appeared to be dual site inhibitors. In particular, **2b** and **2c** fitted this secondary site very well because of their larger 5-arylidene moieties and they were able to perform favourable hydrophobic interactions with a number of lipophilic amino acid side chains lining the pocket: Ala27, Met258, Gly259 and Arg24 (side chain centrepiece). An additional interaction via hydrogen bond between Arg24 and the ether oxygen connecting the terminal phenyl rings of compounds **2b** and **2c** was possible (Figs. 3 and 4). On the other hand, compound **2j** (for which docking positions with the opposite carboxylic group facing Arg221 were also found) achieved positions where hydrogen bonds

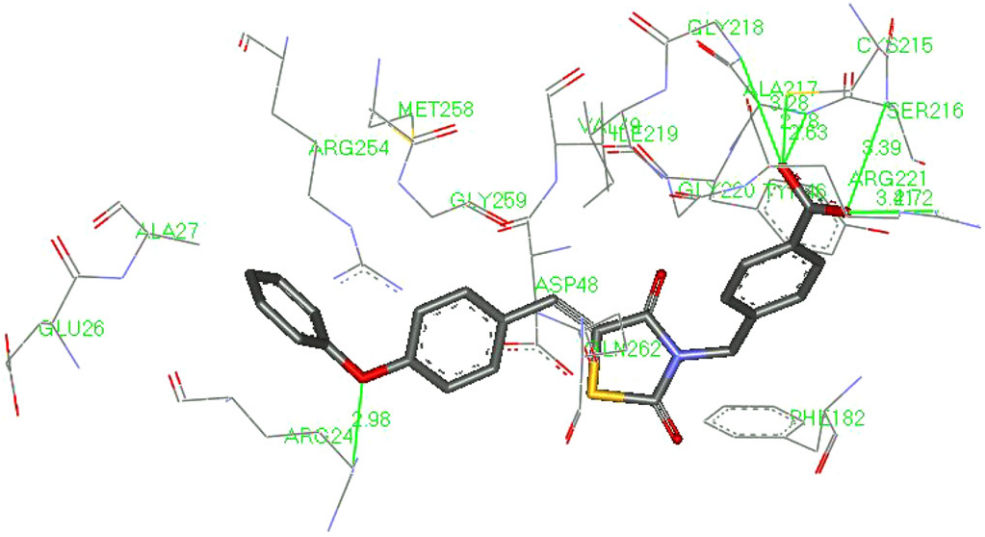


Figure 3. Compound **2b** docked in the PTP1B active site.

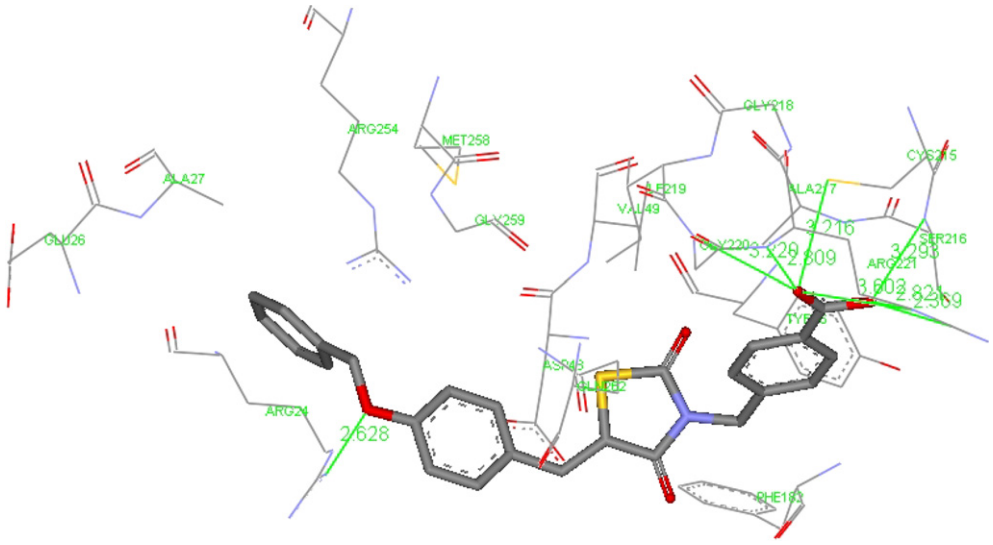


Figure 4. Compound **2c** docked in the PTP1B active site.

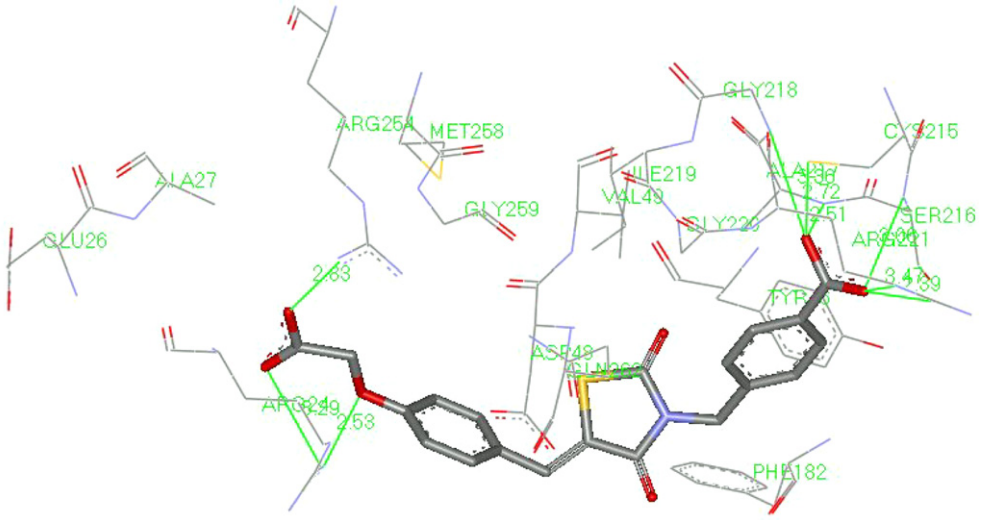


Figure 5. Compound **2j** docked in the PTP1B active site.

and charge–charge interactions between the second carboxylic group and Arg24, Arg254 were possible (Fig. 5). However, **2j** was not able to fill the secondary binding site as well as **2b** and **2c**, due to the smaller size of its 5-arylidene moiety. At the same time, the removal of the second aromatic ring brought about a significant decrease in lipophilicity and in capability to perform additional favourable hydrophobic interactions. This might explain the lower activity of this compound.

5. Conclusions

The design and synthesis of 4-(5-arylidene-2,4-dioxothiazolidin-3-yl)methylbenzoic acids **2** are an example of utilizing pTyr-mimetics to identify effective low molecular weight nonphosphorus monoanionic PTP inhibitors.

Compounds **2b**–**g** were shown to possess PTP1B inhibitory activity in the low micromolar range. Out of these, acids **2b** and **2c** were the most effective inhibitors of both human PTP1B and IF1 isoform of human LMW-PTP, showing from 3- to 38-fold selectivity for PTP1B/IF1 over IF2, Ltp1 and LAR-PTP. In contrast, their inhibition levels against PTP1B, TC-PTP, YopH and PTPβ were very similar.

All tested acids **2** proved to inhibit preferentially PTP1B and IF1 isoform rather than IF2 and Ltp1.

The PTP inhibition data here reported confirmed the initial hypothesis that *p*-methylbenzoic acid residue inserted onto N-3 of a 5-arylidene-2,4-thiazolidinedione scaffold can act as a monoanionic pTyr-mimetic group. The docking simulations into the PTP1B active site indicated that this moiety can replicate the interactions of pTyr with the catalytic site of the enzyme.

In line with the in vitro inhibition and selectivity results, the computational study highlighted that the 5-arylidene moiety of compounds **2b** and **2c** could further interact with aminoacidic residues lining the PTP1B secondary noncatalytic binding pocket. In contrast, 5-(4-carboxymethoxybenzylidene) substituted analogue **2j** was unable to fit this site successfully.

The investigation of structure/activity relationships relevant to this new class of PTP inhibitors has so far been limited to a small library of analogues. However, it emerged that the 5-arylidene moiety markedly influenced their potency and selectivity. A larger lipophilic arylidene moiety containing two aromatic rings appeared to be more favourable than a smaller one composed of a benzylidene ring with hydrophilic substituents.

In conclusion, compounds **2b** and **2c**, which proved to be effective micromolar in vitro inhibitors of both human PTP1B and LMW-PTP, can be used as good low molecular weight, nonpeptide and nonphosphorus starting points for a drug discovery programme aimed at enhancing their potencies and selectivity.

6. Experimental

6.1. Chemistry

Melting points were recorded on a Kofler hot-stage apparatus and are uncorrected. TLC controls were carried out on precoated silica gel plates (F 254 Merck). Elemental analyses (C, H, N), determined by means of a C. Erba mod. 1106 elem. Analyzer, were within ± 0.4% of theory. IR spectra were obtained with a Perkin-Elmer 683 spectrophotometer as Nujol or esachlorobutadiene mulls. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 300 magnetic resonance spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C). Chemical shifts are given in δ units (ppm) relative to internal standard Me_4Si and refer to DMSO-d_6 solutions. Coupling constants (J) are given in hertz (Hz). ^{13}C NMR spectra were determined by Attached Proton Test (APT) experiments and the resonances were always attributed by proton–carbon heteronuclear chemical shift correlation.

Unless stated otherwise, all materials were obtained from commercial suppliers and used without further purification.

6.2. General method for the synthesis of 4-(5-arylidene-2,4-dioxothiazolidin-3-yl) methylbenzoic acids 2a–g

A mixture of 5-arylidene-2,4-thiazolidinedione **1** (10 mmol), 4-(bromomethyl)benzoic acid (4.30 g, 20 mmol) and potassium carbonate (5.53 g, 40 mmol) in acetone (150 ml) was refluxed for 48 h. The solvent was evaporated under reduced pressure and the solid residue was dissolved in methanol; the solution was acidified (pH 3) with HCl and was stirred at room temperature for 30 min. After evaporation to dryness in vacuo, the crude solid was washed with H_2O and recrystallized from methanol providing pure acid **2**.

6.3. 4-[2,4-Dioxo-5-(3-phenoxybenzylidene)thiazolidin-3-yl]methylbenzoic acid (2a)

Yield 54%; mp 207 °C; ^{1}H NMR (DMSO-d_6): δ 4.90 (s, 2H, CH_2); 7.11 (m, 2H, arom); 7.15–7.26 (m, 3H, arom); 7.42–7.49 (m, 5H, arom); 7.60 (m, 1H, arom); 7.93 (m, 2H, arom); 7.98 (s, 1H, CH); ^{13}C NMR (DMSO-d_6): δ 44.4 (CH_2); 119.2 (5-C); 118.9, 120.3, 122.0, 124.1, 124.8, 127.5, 129.6, 130.2, 131.0 (CH arom); 132.8 (CH methylidene); 134.7, 140.1, 155.7, 157.5 (Cq arom); 165.2, 166.8, 167.0 (CO); Anal. ($C_{24}H_{17}NO_5S$) C, H, N.

6.4. 4-[2,4-Dioxo-5-(4-phenoxybenzylidene)thiazolidin-3-yl]methylbenzoic acid (2b)

Yield 50%; mp 252 °C; ^{1}H NMR (DMSO-d_6): δ 4.91 (s, 2H, CH_2); 7.11–7.15 (m, 4H, arom); 7.23 (m, 1H, arom); 7.41–7.49 (m, 4H, arom); 7.67 (m, 2H, arom); 7.92 (m, 2H, arom); 7.97 (s, 1H, CH); ^{13}C NMR (DMSO-d_6): δ 44.4 (CH_2); 119.3 (5-C); 118.2, 119.8, 124.6, 127.5, 129.6, 130.2, 132.4 (CH arom); 133.0 (CH methylidene); 130.1, 140.2, 155.0, 159.1 (Cq arom); 165.5, 166.9, 167.3 (CO); Anal. ($C_{24}H_{17}NO_5S$) C, H, N.

6.5. 4-[5-(4-Benzyloxybenzylidene)-2,4-dioxothiazolidin-3-yl]methylbenzoic acid (2c)

Yield 90%; mp 250 °C (dec); ^{1}H NMR (DMSO-d_6): δ 4.92 (s, 2H, NCH_2); 5.21 (s, 2H, OCH_2); 7.21 (m, 2H, arom); 7.38–7.50 (m, 7H, arom); 7.62 (m, 2H, arom); 7.93 (m, 2H, arom); 7.95 (s, 1H, CH); ^{13}C NMR (DMSO-d_6): δ 44.3 (NCH_2); 69.5 (OCH_2); 115.7, 127.5, 127.8, 128.0, 128.5, 129.6, 132.3 (CH arom); 117.8 (5-C); 133.5 (CH methylidene); 125.5, 130.2, 136.4, 140.3, 160.3 (Cq arom); 165.6, 166.9, 167.4 (CO); Anal. ($C_{25}H_{19}NO_5S$) C, H, N.

6.6. 4-[5-(3-Benzyloxybenzylidene)-2,4-dioxothiazolidin-3-yl]methylbenzoic acid (2d)

Yield 30%; mp 222 °C (dec); ^{1}H NMR (DMSO-d_6): δ 4.84 (s, 2H, NCH_2); 5.17 (s, 2H, OCH_2); 7.15–7.25 (m, 5H, arom); 7.36–7.51 (m, 6H, arom); 7.89 (m, 2H, arom); 7.96 (s, 1H, CH); ^{13}C NMR (DMSO-d_6): δ 44.0 (NCH_2); 68.9 (OCH_2); 115.6, 121.0, 121.8, 127.1, 127.3, 127.8, 128.8, 132.7, 135.7 (CH arom); 117.0 (5-C); 133.7 (CH methylidene); 126.0, 129.9, 136.1, 138.1, 158.1 (Cq arom); 164.8, 166.6, 168.9 (CO); Anal. ($C_{25}H_{19}NO_5S$) C, H, N.

6.7. 4-(5-Naphthalen-1ylmethylene-2,4-dioxothiazolidin-3-yl)methylbenzoic acid (2e)

Yield 60%; mp 268 °C; ^{1}H NMR (DMSO-d_6): δ 4.93 (s, 2H, CH_2); 7.45 (m, 2H, arom); 7.57–7.73 (m, 4H, arom); 7.92 (m, 2H, arom); 8.02–8.14 (m, 3H, arom); 8.59 (s, 1H, CH); ^{13}C NMR (DMSO-d_6): δ 44.3 (CH_2); 123.4, 125.5, 126.4, 126.8, 127.4, 127.6, 128.8, 129.6, 130.5 (CH arom); 124.7 (5-C); 131.0 (CH methylidene);

130.1, 130.2, 130.8, 133.2, 140.1 (Cq arom); 166.9, 167.1, 167.6 (CO); Anal. ($C_{22}H_{15}NO_4S$) C, H, N.

6.8. 4-(5-Naphthalen-2ylmethylene-2,4-dioxothiazolidin-3-yl)methylbenzoic acid (2f)

Yield 72%; mp 288–290 °C (dec); ^{1}H NMR (DMSO-d_6): δ 4.92 (s, 2H, CH_2); 7.44 (m, 2H, arom); 7.59–7.76 (m, 3H, arom); 7.92–8.09 (m, 4H, arom); 8.14 (m, 2H, arom); 8.26 (s, 1H, CH); ^{13}C NMR (DMSO-d_6): δ 44.5 (CH_2); 121.3 (5-C); 126.2, 127.2, 127.6, 127.7, 128.6, 128.7, 129.7, 130.1, 133.6 (CH arom); 131.3 (CH methylidene); 130.5, 131.6, 132.8, 132.9, 140.3 (Cq arom); 165.6, 167.0, 167.5 (CO); Anal. ($C_{22}H_{15}NO_4S$) C, H, N.

6.9. 4-[5-(4-Hydroxy-3-methoxybenzylidene)-2,4-dioxothiazolidin-3-yl]methylbenzoic acid (2g)

This was synthesized as above described, adding 4-(bromomethyl)benzoic acid in small amounts over 2 h to a solution of compound **1g** and potassium carbonate in acetone. Yield 34%; mp 242 °C; ^{1}H NMR (DMSO-d_6): δ 3.80 (s, 3H, OCH_3); 4.88 (s, 2H, CH_2); 6.92 (d J = 8.4, 1H, arom); 7.11 (dd J = 8.4 and 1.8, 1H, arom); 7.20 (d J = 1.8, 1H, arom); 7.39 (m, 2H, arom); 7.88 (s, 1H, CH); 7.90 (m, 2H, arom); 10.04 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6): δ 44.3 (NCH_2); 55.7 (OCH_3); 113.7, 124.3, 127.6, 129.3, 130.3 (CH arom); 116.6 (5-C); 134.2 (CH methylidene); 126.1, 140.4, 141.6, 148.1, 149.9 (Cq arom); 165.6, 167.0, 167.5 (CO); Anal. ($C_{19}H_{15}NO_6S$) C, H, N.

6.10. 4-[5-(4-Hydroxybenzylidene)-2,4-dioxothiazolidin-3-yl]methylbenzoic acid (2h)

A mixture of 4-[5-(4-benzyloxybenzylidene)-2,4-dioxothiazolidin-3-yl]methylbenzoic acid (**2c**) (4.45 g, 10 mmol), glacial AcOH (120 ml) and HCl 12 N (30 ml) was refluxed for 2 h. After evaporation in vacuo, the residue was refluxed again with AcOH (120 ml) and HCl (30 ml) for 2 h. After evaporation to dryness in vacuo, the crude solid was washed with H_2O and recrystallized from ethanol providing pure acid **2h**. Yield 85%; mp 290 °C; ^{1}H NMR (DMSO-d_6): δ 4.89 (s, 2H, CH_2); 6.92 (m, 2H, arom); 7.40 (m, 2H, arom); 7.52 (m, 2H, arom); 7.88 (s, 1H, CH); 7.92 (m, 2H, arom); 10.40 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6): δ 44.2 (NCH_2); 116.4 (5-C); 116.5, 127.5, 129.7, 132.7 (CH arom); 134.1 (CH methylidene); 123.8, 130.3, 140.3, 160.3 (Cq arom); 165.6, 166.9, 167.5 (CO); Anal. ($C_{18}H_{13}NO_5S$) C, H, N.

6.11. 4-[5-(3-Hydroxybenzylidene)-2,4-dioxothiazolidin-3-yl]methylbenzoic acid (2i)

The procedure was the same as reported for compound **2h**, starting from 4-[5-(3-benzyloxybenzylidene)-2,4-dioxothiazolidin-3-yl]methylbenzoic acid (**2d**). Yield 38%; mp 260 °C (dec); ^{1}H NMR (DMSO-d_6): δ 4.86 (s, 2H, CH_2); 6.90 (m, 1H, arom); 7.01 (s, 1H, arom); 7.08 (m, 1H, arom); 7.34 (m, 1H, arom); 7.36 (m, 2H, arom); 7.87 (s, 1H, CH); 7.88 (m, 2H, arom); 9.89 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6): δ 44.6 (NCH_2); 115.8, 117.9, 121.2, 127.3, 129.6, 130.5 (CH arom); 120.8 (5-C); 133.7 (CH methylidene); 130.4, 133.8, 140.4, 157.9 (Cq arom); 165.5, 166.9, 167.4 (CO); Anal. ($C_{18}H_{13}NO_5S$) C, H, N.

6.12. 4-[5-(4-Carboxymethoxybenzylidene)-2,4-dioxothiazolidin-3-yl]methylbenzoic acid (2j)

A mixture of 4-[5-(4-hydroxybenzylidene)-2,4-dioxothiazolidin-3-yl]methylbenzoic acid **(2h)** (3.55 g, 10 mmol), methyl bromoacetate (3.06 g, 20 mmol) and potassium carbonate (2.76 g, 20 mmol) in acetone (120 ml) was refluxed for 24 h. After cooling, the inorganic salts were filtered off; then the solvent was evaporated under reduced pressure. A mixture of the solid residue (3.93 g), glacial AcOH (120 ml) and HCl 12 N (30 ml) was refluxed for 2 h. After evaporation in vacuo, the residue was refluxed again with AcOH (120 ml) and HCl (30 ml) for 2 h. After evaporation to dryness in vacuo, the crude solid was washed with H_2O and recrystallized from ethanol providing pure acid **2j**. Yield 84%; mp 280 °C; ^{1}H NMR (DMSO-d_6): δ 4.75 (s, 2H, NCH_2); 4.90 (s, 2H, OCH_2); 7.09 (m, 2H, arom); 7.42 (m, 2H, arom); 7.61 (m, 2H, arom); 7.91 (m, 2H, arom); 7.94 (s, 1H, CH); ^{13}C NMR (DMSO-d_6): δ 44.9 (NCH_2); 65.2 (OCH_2); 116.1, 128.1, 130.2, 132.8 (CH arom); 118.7 (5-C); 134.0 (CH methylidene); 126.4, 130.8, 140.9, 160.3 (Cq arom); 166.1, 167.5, 167.8 (CO); Anal. ($C_{20}H_{15}NO_7S$) C, H, N.

7. Molecular modelling

Docking calculations were performed on an SGI Octane double processor R10K workstation using the LigandFit module in the Cerius[2] software package version 4.10[49] and the cff1.02 forcefield. For ligand minimisation the software tool Catalyst version 4.10[50] was used. Site definition resulted from the cocrystallized ligand in the PDB complex 1XBO.[31] Flexible docking runs were carried out without interaction filters and all scoring functions implemented in Cerius[2] served for ranking analyses. For site definition, docking, and scoring calculations, default parameter settings were kept, if not mentioned otherwise in the text. In the docking preferences a fixed number of Monte Carlo trials of 5000, a maximum number of 20 saved poses, and 500 iterations in the final rigid body minimisation were required, the force field selection box in the energy preferences was set to CFF.

8. Enzyme section

The complete coding sequences of IF1, IF2, Ltp1 LMW-PTPs and PTP1B were cloned in frame with the sequence of the glutathione S-transferase (GST) in the pGEX-2T bacterial expression vector. Enzyme expression and purification were achieved in the *E. coli* TB1 strain.[51] Briefly, the recombinant fusion proteins were purified from bacterial lysate using a single-step affinity chromatography. The solution containing purified fusion proteins was treated with thrombin for 3 h at

37°C. Then the enzymes were purified from GST and thrombin by gel filtration. The purity of proteins' preparations was analysed by SDS–polyacrylamide gel electrophoresis according to Laemmli.[52] TC-PTP, LAR-PTP, PTPβ and YopH were purchased from SIGMA.

9. Enzymatic assay and inhibition experiments

The enzymatic assay was carried out at 37 °C using *p*-nitrophenylphosphate as substrate; the final volume was 1 ml. The assay buffer (pH 7.0) contained 0.075 M of β,β-dimethylglutarate buffer, 1 mM EDTA and 5 mM dithiothreitol. The reactions were initiated by addition of aliquots of the enzyme preparations and stopped at appropriate times by adding 4 ml of 1 M KOH. The released *p*-nitrophenol was determined by reading the absorbance at 400 nm ($\varepsilon = 18{,}000\ M^{-1}\ cm^{-1}$). The main kinetic parameters (K_m and V_{max}) were determined by measuring the initial rates at different substrate concentrations. Experimental data were analysed using the Michaelis equation and a nonlinear fitting program (FigSys).[53]

Inhibition constants were determined measuring initial hydrolysis rates at differing substrate and inhibitor concentrations. The apparent K_m values measured at the various inhibitor concentrations were plotted against concentration of the inhibitor to calculate the K_i values. All initial rate measurements were carried out in triplicate. For each inhibitor, IC_{50} was determined by measuring the initial hydrolysis rate under fixed *p*-nitrophenylphosphate concentration, equal to the K_m value of the considered PTP. Data were fitted to the following equation using the FigSys program: $V_i/V_0 = IC_{50}/(IC_{50} + [I])$, where V_i is the reaction velocity when the inhibitor concentration is [I], V_0 is the reaction velocity with no inhibitor and $IC_{50} = K_i + K_i[S]/K_m$. Therefore, when the substrate concentration [S] is equal to K_m, $IC_{50} = 2K_i$.

Preliminarily, the solubility and stability of the tested compounds were evaluated. Compounds **2** are soluble in DMSO/H_2O $\leqslant$5% or CH_3OH/ H_2O = 5% mixtures. They proved to be stable under the same experimental conditions of the enzymatic assay.

10. Inhibition assays with a phosphopeptide substrate

The protein tyrosine phosphatase assay kit was purchased from Sigma. The [pTyr^{1146}]phosphorylated-peptide TRDIpYETDYYRK, corresponding to the sequence 1142–1153 of the insulin receptor, was used as substrate for the inhibition assays.

All assays were performed at 37 °C in triplicate in a 96-well microtitre plate. The peptide TRDIpYETDYYRK was dissolved in 0.075 M β,β-dimethylglutarate buffer, pH 7.0, containing 1 mM dithiothreitol and 1 mM EDTA. The final volume was 50 μl; the reaction was started by adding an aliquot of PTP1B (40 nM final concentration) and stopped by adding 50 μl of Malachite Green/ammonium molybdate reagent solution. The amount of inorganic phosphate released from the peptide was determined reading the absorbance at 595 nm. Control assays without PTP1B were also performed. The K_m and V_{max} of PTP1B for the phosphopeptide were determined measuring the rate of inorganic phosphate release at increasing substrate concentration ranging from 10 to 400 μM.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.05.027.

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